Gluconeogenesis from D-Glyceraldehyde and Dihydroxyacetone in Isolated Rat Liver. Stimulation by Glucagon†

Carlo M. Veneziale

With the Technical Assistance of Michael E. Nesheim

ABSTRACT: Glucagon can stimulate the conversion of D-glyceraldehyde and dihydroxyacetone into glucose in isolated livers (from fasted rats) which have been preperfused with quinolinate. Quinolinate, an inhibitor of phosphoenolpyruvate carboxykinase, was used to inhibit gluconeogenesis from lactate and other precursors requiring that enzyme. During the 15 min following D-glyceraldehyde addition (4.0 mM), mean glucose formation was 92 μmoles/10 g of liver (SD, 16; *N*, 20) without glucagon and 167 μmoles/10 g (SD, 24; *N* 14) when glucagon was added. During the 15 min following dihydroxyacetone addition (4.0 mM), mean glucose formation was 114 μmoles/10 g (SD, 19; *N*, 13) without glucagon and 175 μmoles/10 g (SD, 23; *N*, 6) with it. Glycogen measurements showed that stimulation of glucose release by glucagon could not be accounted for by glycogenolysis. The effect of glucagon was to stimulate gluconeogenesis by 45 μmoles/10 g per 15 min when D-glyceraldehyde was substrate and by approximately 35 μmoles/10 g per 15 min when dihydroxyacetone was substrate. In contrast, gluconeogenesis from glycerol (4.0 mM) was unstimulated by the hormone. The results tend to confirm previously reported data on the glucagon stimulation of glucose formation from fructose (Veneziale, C. M. (1971), *Biochemistry 10*, 3443). Measurements of various hepatic intermediates in freeze-clamped tissue specimens did not disclose the site of glucagon stimulation in the gluconeogenic pathway of the trioses. Possible site(s) of action and the potential physiologic significance of these observations are discussed.

The discovery that pancreatic glucagon can be inappropriately secreted (Unger *et al.*, 1970) makes it of potential medical importance that the site(s) and mechanism(s) of action of the hormone on hepatic gluconeogenesis be fully understood. In the studies reported in this paper, glucagon stimulated gluconeogenesis from D-glyceraldehyde and dihydroxyacetone but not from glycerol. Preperfusion with quinolinate permitted study of gluconeogenesis from the added substrates under conditions in which gluconeogenesis from newly formed lactate and from many endogenous precursors was inhibited. The results presented here tend to confirm previously reported data on the glucagon stimulation of glucose formation from fructose (Veneziale, 1971).

Materials and Methods

The livers used in the perfusion experiments were taken from male Sprague–Dawley rats (300–400 g) that had been fasted for 24 hr. The perfusate consisted of twice-washed bovine erythrocytes suspended (hematocrit value, 30%) in Krebs–Ringer bicarbonate solution (Umbreit *et al.*, 1964) containing 3% (w/v) bovine albumin (Schwarz/Mann, Fraction V powder). The first 20 ml of perfusate leaving the liver was discarded. The final perfusate volume in our recirculating system (Veneziale *et al.*, 1967) was 100 ml, and the flow rate was maintained at 1 ml/g wet weight of liver.

Glucose was assayed enzymatically as described by Slein (Bergmeyer, 1965) in 1:20 filtrates of perfusate prepared by the method of Somogyi (1945). Liver glycogen was isolated by the method of Good *et al.* (1933) which was scaled down to smaller volumes in order to minimize glycogen loss due to its limited solubility in 60% ethanol (Kerly, 1930; Walaas and Walaas, 1950). Precipitation from 60% ethanol was facilitated by centrifuging at 2600*g* for 30 min at 0–5°. After one wash in 60% ethanol, the glycogen was hydrolyzed with 4.0 ml of 1.0 N HCl at 100° for 2 hr; the glucose released was assayed by the method of Slein. When this method was applied to standard glycogen samples weighing 150–400 μg, the mean recovery was 89% (SD, 5; *N*, 14).

The specimens of liver (500–1000 mg) snipped from the organ being perfused were weighed on a torsion balance (30–40 sec) prior to immersion into 1.0 ml of hot 30% KOH. To determine if the 30-sec delay resulted in significant net breakdown of glycogen, the largest lobe of liver from each of six anesthetized rats was sampled. Half of the specimen was instantly clamped at the temperature of liquid N_2, then powdered, and weighed in the frozen state. The other half was weighed at room temperature. Both the frozen powders and the liver specimens were digested and processed as described above. The mean content of glycogen (as μmoles of glucose per 10 g wet weight of liver) in those subjected to freeze clamping was 218 (SD, 141; *N*, 6; range 48–409); in those not frozen the mean was 19% less (SD, 8; *N*, 6). The difference, if real, is relatively small and would not alter any of the conclusions drawn from the data given in this paper. More pertinent to this work is our observation that the two methods gave the same results when the glycogen content was no greater than 40 μmoles of glucose/10 g of liver, which is the usual finding in the perfused liver from the 24-hr fasted rat (Hems *et al.*, 1966; Exton and Park, 1968; Menahan *et al.*, 1968).

D-Glyceraldehyde (Schwarz/Mann), dihydroxyacetone (Nutritional Biochemicals), and glycerol (Fisher) were of the

† From the Mayo Clinic and Mayo Foundation, Department of Endocrine Research, Rochester, Minnesota 55901. *Received February 24, 1972.* This paper is IV in the series: Hepatic Gluconeogenesis. This investigation was supported by grants from the Mayo Foundation, National Science Foundation, American Diabetes Association, and Eli Lilly and Company. Crystalline glucagon (lot no. 258-234B-167-1) was supplied by Eli Lilly and Company.

highest purity obtainable. To assay D-glyceraldehyde and dihydroxyacetone in aqueous solution, the compounds were reduced to glycerol with sodium borohydride at neutral pH. The reducing agent was oxidized by acidification, and, after the pH was adjusted, the newly formed glycerol was phosphorylated by glycerokinase (Boehringer-Mannheim) and ATP. The ADP formed was then assayed by the method of Adam (Bergmeyer, 1965).

D-Glyceraldehyde and dihydroxyacetone solutions were stored frozen; 0.2–0.4 M solutions were demonstrated to be stable for at least 4 weeks. The use of freshly prepared D-glyceraldehyde solutions was avoided to ensure complete hydrolysis of the glyceraldehyde dimer (Wohl, 1898; for a discussion, see Landau and Merlevede, 1963).

The radiochemical purity of the D-glyceraldehyde-*U*-^{14}C (New England Nuclear) was approximately 80%. Its separation from labeled impurities was achieved by thin-layer chromatography on silica gel with methylene chloride–methanol–0.01 N HCl, 40:20:3 (v/v/v). Localization was by radioautography. After elution of the most radioactive band with water, its identity as D-glyceraldehyde-*U*-^{14}C was indicated as follows: (1) by assay for D-glyceraldehyde, (2) by demonstrating mobility on thin-layer chromatography identical with that of unlabeled standard in two different solvent systems, and (3) by forming the 2,4-dinitrophenylhydrazone and demonstrating on thin-layer plates that the radioactivity migrated with the derivative.

Protein-free perchloric acid extracts of perfusates (4.0 ml equivalent to 1.0 ml of perfusate) were neutralized with K_2CO_3. For chromatography on paper by system 2 of Veneziale and Gabrielli (1969) to isolate glucose-^{14}C formed from labeled substrate, 0.5 ml of ice-cold extract was evaporated under a stream of N_2 to approximately 50 μl. This concentrate was then quantitatively transferred to the origin in 5-μl aliquots, followed by two water washes of 20 μl each. The area to which glucose-^{14}C had migrated was located by radioautography, cut out, and counted in a toluene-based scintillation liquid (Veneziale, 1971).

During perfusion with quinolinate and D-glyceraldehyde, liver specimens were freeze clamped at the temperature of liquid N_2. The frozen powder was weighed, deproteinized, and extracted with 6% perchloric acid ($HClO_4$). Sonication, rather than homogenization, of the $HClO_4$-soaked powder was found to be an efficient step in the extraction procedure, which was conventional in every other way (Bergmeyer, 1965). The protein-free aqueous extract was neutralized to pH 5 before being made to volume with water (1.0 g of tissue equivalent to 8.0 ml of extract). Recovery of standards of the main metabolites to be measured was 100% by this procedure. Intermediates were determined enzymatically as described in Bergmeyer (1965): glucose 6-phosphate and fructose 6-phosphate by the method of Hohorst; fructose diphosphate and dihydroxyacetone phosphate by the method of Bücher and Hohorst; α-L-glycerophosphate by the method of Hohorst; glycerol by the method of Wieland; lactate by the method of Hohorst; ATP, pyruvate, ADP, and AMP by the methods of Adam.

Results

Rates of Glycogenesis. The rates were similar regardless of substrate (Table I). Glycogen was 16% of the total carbohydrate formed with D-glyceraldehyde as substrate (see Table II for amounts of glucose released), 11% with dihydroxyacetone, and 12% with glycerol. The extent of glycogenolysis caused by glucagon (−12 ± 5 μmoles/10 g per 15 min) was predictable on the basis of the 60-min hepatic glycogen content (17 ± 8 μmoles/10 g; *N*, 17).

TABLE I: Glycogen Formation as Glucose.[a]

Substrate[b]	*N*[c]	Glucose (μmoles/10 g per 15 min)[d]
D-Glyceraldehyde	11	18 ± 12
Dihydroxyacetone	8	14 ± 10
Glycerol	6	18 ± 11
D-Glyceraldehyde plus glucagon	5	−12 ± 5

[a] See Table II for description of experimental conditions. [b] Concentration in perfusate, 4.0 mM. [c] Number of determinations. [d] Means (±SD) of increments found in individual livers 15 min after addition of substrate to perfusate.

Rates of Hepatic Glucose Release. Glucagon caused an increase in the rate of glucose release in the presence of D-glyceraldehyde at both 2.0 and 4.0 mM (Table II) and caused a definite increase in gluconeogenesis at 4.0 mM. Considering only the 4.0 mM series, the total carbohydrate formed—represented by that released and that converted to glycogen—was 92 + 18 μmoles/10 g per 15 min. In the presence of glucagon, the rate of formation of new carbohydrate was that released minus that due to glycogenolysis: 167 − 12 μmoles/10 g per 15 min. Therefore, the effect of glucagon was to stimulate gluconeogenesis by 45 μmoles/10 g per 15 min ($P < 0.005$) (Sheffe, 1970). The increased glucose release seen with 4.0 mM D-glyceraldehyde was not seen with 6.0 mM; the reason for this is unknown. From the dihydroxyacetone data, the augmentation was 35 μmoles/10 g per 15 min ($P < 0.025$). In contrast gluconeogenesis from glycerol was unstimulated by the hormone. When one considers the glycerol data of Tables I and II, the total carbohydrate formed was 134 + 18 μmoles/10 g per 15 min (*cf.* 162 μmoles/10 g per 15 min in Table II) which is only 10 μmoles less than the amount released in the presence of glucagon; the difference can be attributed to glycogenolysis.

Perfusion with D-*Glyceraldehyde-U-*^{14}C. The isotope data (Table III) demonstrate that the chemical measurement data of Table II reflect primarily the metabolism of added glyceraldehyde. The increase of ^{14}C incorporation in the presence of glucagon roughly parallels the increase in amount of glucose.

Hepatic Metabolites and Adenine Nucleotides during Perfusion with D-*Glyceraldehyde.* There were no differences due to glucagon in the concentrations of glycerol and the phosphorylated intermediates (Table IV), with the possible exception of a slightly higher glucose 6-phosphate concentration relative to fructose 6-phosphate as reported previously (Veneziale, 1971). It is noteworthy that our data do not show an accumulation of any metabolite, including glyceraldehyde 3-phosphate, due to deinhibition of a rate-limiting reaction by glucagon. This fact taken together with the glycerol data of Table II suggests the absence of an effect of glucagon on glucose 6-phosphatase, phosphoglucoisomerase, fructose diphosphatase, aldolase, and phosphotrioseisomerase. As a result of glucagon addition, there was a decrease in the concentrations of lactate and pyruvate, as noted previously in similar experiments in which fructose was used as substrate

TABLE II: Rates of Hepatic Glucose Release.[a]

Substrate (Perfusate Concentration)	Without Glucagon (μmoles/10 g)		With Glucagon (μmoles/10 g)	
	Per 10 min	Per 15 min	Per 10 min	Per 15 min
None[b]	13 ± 6 (3)	25 ± 6 (5)	31 ± 13 (3)	40 ± 12 (5)
D-Glyceraldehyde (2.0 mM)	52 ± 9 (8)	68 ± 15 (10)	72 ± 12 (5)	107 ± 11 (5)
D-Glyceraldehyde (4.0 mM)[c]	61 ± 25 (7)	92 ± 16 (20)	117 ± 21 (7)	167 ± 24 (14)[d]
D-Glyceraldehyde (6.0 mM)	83 ± 25 (3)	108 ± 17 (3)	97 ± 26 (3)	141 ± 21 (3)
Dihydroxyacetone (4.0 mM)	87 ± 17 (6)	114 ± 19 (13)	123 ± 20 (6)	175 ± 23 (6)
Glycerol (4.0 mM)		134 ± 27 (12)		162 ± 16 (5)

[a] Quinolinate was added to the perfusate at 30 min to give a final perfusate concentration of 2.4 mM; substrates and glucagon were added at 60 min. The mean perfusate glucose content at 60 min was 236 μmoles (SD, 23; *N*, 12), which was representative of the entire series. Bovine erythrocytes were shown not to metabolize D-glyceraldehyde. [b] Additional control data are reported elsewhere (Veneziale, 1971). [c] Liver mean weight was 7.7 g (SD, 1.0; *N*, 25). [d] When N^6-monobutyryladenosine 3′,5′-monophosphate in cyclic form (1×10^{-5} M) was used instead of glucagon, the results were 139 ± 12 (4).

TABLE III: Glucose-^{14}C Formation from D-Glyceraldehyde-*U*-^{14}C.[a]

Interval Perfused (min)	No Glucagon		Glucagon (5 μg)	
	μmoles	× 10^6 Dpm	μmoles	× 10^6 Dpm
60–70	46	3.9	103	7.3
60–70	62	4.4	118	7.3
60–75	90	6.6	150	10.7
60–75	91	6.0	165	9.5

[a] Quinolinate (2.4 mM) was added at 30 min. Substrate (4.0 mM; 9 μCi) and glucagon were added at 60 min. Data shown as per 10 g of liver. Dpm values are in total circulating perfusate glucose at the end of designated interval.

(Veneziale, 1971). Glucagon did not, however, alter the ratio of lactate to pyruvate, indicating that its action on D-glyceraldehyde metabolism must be independent of the oxidation–reduction potential of the extramitochondrial NAD^+:NADH couple. The intracellular concentration of glyceraldehyde 4 and 8 min after its addition to the perfusate was 0.38 μmole/g (SD, 0.09; *N*, 6) in the absence of hormone and 0.33 (SD, 0.10; *N*, 5) in its presence.

Clearance Data on D-Glyceraldehyde. A significant effect of glucagon on D-glyceraldehyde transport into liver is not suggested by the data (Table V). The action of glucagon in stimulating gluconeogenesis from D-glyceraldehyde must have to do with an intracellular event of D-glyceraldehyde metabolism. This was established when it was demonstrated that the hormone stimulated gluconeogenesis from fructose but not the clearance of fructose (Veneziale, 1971), which is metabolized to D-glyceraldehyde in liver.

TABLE IV: Hepatic Metabolites and Adenine Nucleotides during Perfusion with D-Glyceraldehyde.[a]

Compound	Concentration (μmoles/g Wet Wt)			
	No Glucagon		Glucagon (5 μg)	
	4 min[b]	8 min[b]	4 min[b]	8 min[b]
Lactate	0.46 ± 0.18 (5)	0.53 ± 0.06 (6)	0.23 ± 0.08 (5)	0.28 ± 0.06 (4)
Pyruvate	0.22 ± 0.08 (4)	0.30 ± 0.02 (4)	0.10 ± 0.04 (4)	0.16 ± 0.04 (3)
Glycerol	0.014 ± 0.011 (5)	0.018 ± 0.019 (6)	0.013 ± 0.016 (5)	0.017 ± 0.020 (4)
α-L-Glycerophosphate	0.087 ± 0.033 (5)	0.068 ± 0.033 (6)	0.076 ± 0.032 (5)	0.091 ± 0.026 (5)
Dihydroxyacetone phosphate	0.021 ± 0.005 (5)	0.023 ± 0.009 (6)	0.015 ± 0.004 (5)	0.018 ± 0.006 (5)
Fructose diphosphate[c]	0.0086 ± 0.0037 (5)	0.0080 ± 0.0022 (6)	0.0078 ± 0.0033 (5)	0.0086 ± 0.0036 (5)
Fructose 6-phosphate	0.020 ± 0.002 (5)	0.016 ± 0.004 (6)	0.020 ± 0.011 (5)	0.016 ± 0.004 (5)
Glucose 6-phosphate	0.044 ± 0.008 (5)	0.037 ± 0.006 (6)	0.071 ± 0.031 (5)	0.054 ± 0.012 (5)
ATP	3.43 ± 0.51 (4)	3.10 ± 0.38 (4)	3.06 ± 0.43 (4)	2.96 ± 0.44 (4)
ADP	1.20 ± 0.47 (4)	1.02 ± 0.19 (4)	1.22 ± 0.11 (4)	1.07 ± 0.09 (4)
AMP	0.34 ± 0.13 (4)	0.29 ± 0.03 (4)	0.35 ± 0.06 (4)	0.34 ± 0.08 (4)

[a] Quinolinate was added at 30 min to give a concentration of 2.4 mM; substrate was added at 60 min to give a concentration of 4.0 mM. [b] Time lapse between substrate addition and tissue sampling. [c] As measured here (see Materials and Methods), the results of this assay indicate no accumulation of glyceraldehyde 3-phosphate.

TABLE V: Disappearance of Added D-Glyceraldehyde (4.0 mM) from Perfusate.[a]

	D-Glyceraldehyde Cleared (μmoles/15 min)		Lactate Released (μmoles/10 g per 15 min)	Rate of Glucose Release (μmoles/10 g per 15 min)	Liver Weight (g)
	Per Whole Liver	Per 10 g of Liver			
No glucagon	268 ± 15 (6)	340 ± 45 (6)	29 ± 13 (6)	94 ± 19 (6)	7.98 ± 1.21 (6)
Glucagon (5 μg)	295 ± 13 (4)	362 ± 67 (4)	17 ± 10 (4)	151 ± 10 (4)	8.30 ± 1.11 (4)

[a] Quinolinate was added at 30 min to give a concentration of 2.4 mM; substrate was added at 60 min to give a concentration of 4.0 mM.

Discussion

The effect of glucagon on gluconeogenesis from D-glyceraldehyde, as demonstrated here, might be considered to be of significance from at least one point of view. For example, if one assumes a similar response in the human (average adult male liver weight, 1500 g), the rate of glucose production augmented by glucagon—as, perhaps, under conditions of inappropriate glucagon secretion (Unger *et al.*, 1970)—could amount to 117 g/24 hr. This estimate is based on a steady supply of substrate, a condition which could be met by the diets of many. In the United States, the average daily intake of fructose derived from sucrose alone is approximately 65 g (Yudkin, 1969a) and this excludes that derived from foods which are rich in fructose as the free monosaccharide (Hardinge *et al.*, 1965). It is relatively common to find people whose sucrose intake is great enough to provide 125–150 g of fructose daily from that source alone (Yudkin, 1969b). And it seems established that the major pathway of fructose metabolism results in D-glyceraldehyde formation (see Heinz *et al.*, 1968, for references).

The complete fate of D-glyceraldehyde in our control livers remains to be determined. At present our data have indicated little lactate and no glycerol accumulation as measured in perfusate. There is a possibility that pyruvaldehyde formation takes place in accordance with the finding of Bonsignore *et al.* (1968), but we have yet to look into this.

The data of this paper together with those of paper II of this series (Veneziale, 1971) suggest an action of glucagon which results in an increase in the rate of conversion of D-glyceraldehyde to phosphorylated intermediates. This could be accomplished at the level of D-triokinase, which catalyzes the formation of glyceraldehyde 3-phosphate. It could also be accomplished by one of the glycerol dehydrogenases which catalyze the formation of glycerol; one requires NADH and the other requires NADPH (Wolf and Leuthardt, 1953; Moore, 1959; Heinz *et al.*, 1968; Sillero *et al.*, 1969). The triokinase would be the most attractive choice for several reasons, including the fact that the enzyme is able to use both D-glyceraldehyde and dihydroxyacetone (Hers, 1962) and this would be in harmony with the results of this paper. These considerations, which as yet are speculative, are under study.

References

Bergmeyer, H.-U. (1965), Methods of Enzymatic Analysis, New York, N. Y., Academic Press, Inc.

Bonsignore, A., Castellani, A., Fornaini, G., Leoncini, G., and Segni, P. (1968), *Ital. J. Biochem. 17*, 65.

Exton, J. H., and Park, C. R. (1968), *J. Biol. Chem. 243*, 4189.

Good, C. A., Kramer, H., and Somogyi, M. (1933), *J. Biol. Chem. 100*, 485.

Hardinge, M. G., Swarner, J. B., and Crooks, H. (1965), *J. Amer. Diet. Ass. 46*, 197.

Heinz, F., Lamprecht, W., and Kirsch, J. (1968), *J. Clin. Invest. 47*, 1826.

Hems, R., Ross, B. D., Berry, M. N., and Krebs, H. A. (1966), *Biochem. J. 101*, 284.

Hers, H. G. (1962), *Methods Enzymol. 5*, 362.

Kerly, M. (1930), *Biochem. J. 24*, 67.

Landau, B. R., and Merlevede, W. (1963), *J. Biol. Chem. 238*, 861.

Menahan, L. A., Ross, B. D., and Wieland, O. (1968), *Biochem. Biophys. Res. Commun. 30*, 38.

Moore, B. W. (1959), *J. Amer. Chem. Soc. 81*, 5837.

Sheffe, H. (1970), *J. Amer. Statist. Ass. 65*, 1501.

Sillero, M. A. G., Sillero, A., and Sols, A. (1969), *Eur. J. Biochem. 10*, 345.

Somogyi, M. (1945), *J. Biol. Chem. 160*, 69.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1964), Manometric Techniques: A Manual Describing Methods Applicable to the Study of Tissue Metabolism, 4th ed, Minneapolis, Minn., Burgess Publishing Company.

Unger, R. H., Aguilar-Parada, E., Müller, W. A., and Eisentraut, A. M. (1970), *J. Clin. Invest. 49*, 837.

Veneziale, C. M. (1971), *Biochemistry 10*, 3443.

Veneziale, C. M., and Gabrielli, F. (1969), *Anal. Biochem. 28*, 206.

Veneziale, C. M., Walter, P., Kneer, N., and Lardy, H. A. (1967), *Biochemistry 6*, 2129.

Walaas, O., and Walaas, E. (1950), *J. Biol. Chem.187*, 769.

Wohl, A. (1898), *Ber. Deut. Chem. Ges. 31*, 2394.

Wolf, H. P., and Leuthardt, F. (1953), *Helv. Chim. Acta 36*, 1463.

Yudkin, J. (1969a), *Nutr. News 32*, 9.

Yudkin, J. (1969b), *Nutr. Today 4*, 16.